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pH Regulation in Mouse Sperm: Identification of Na^{+}-, Cl^{-}-, and HCO_3^{-}-Dependent and Arylaminobenzoate-Dependent Regulatory Mechanisms and Characterization of Their Roles in Sperm Capacitation

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Intracellular pH (pH_i) regulates several aspects of mammalian sperm function, although the transport mechanisms that control pH_i in these cells are not understood. The pH_i of mouse cauda epididymal sperm was determined from the fluorescence excitation ratio of 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein and calibrated with nigericin and elevated external [K^+]. Two acid efflux mechanisms were identified following imposition of acid loads. One pathway has many anticipated characteristics of the somatic Na^+-dependent Cl^-–HCO_3^- exchanger, although sperm and somatic mechanisms can be distinguished by their ion selectivity and inhibitor sensitivity. Sperm may have an isoform of this exchange pathway with novel functional characteristics. The second acid-export pathway does not require extracellular anions or cations and is inhibited by arylaminobenzoates (flufenamic acid, diphenylamine-2-carboxylate). Mouse sperm also recover spontaneously from intracellular alkalinization. Recovery rates in *N*-methyl-D-glucamine$^+$ Cl^- or in 0.25 *M* sucrose are not significantly different from that in a complex culture medium. Thus, recovery from alkalinization does not utilize specific, ion-dependent transport mechanisms. Other widely distributed acid-efflux mechanisms, such as the Na^+–H^+ antiport pathway and the Na^+-independent Cl^-–HCO_3^- exchanger are not major regulators of mouse sperm pH_i. Sperm capacitation results in pH_i increases (from 6.54 ± 0.08 to 6.73 ± 0.09) that require a functional Na^+-, Cl^--, and HCO_3^--dependent acid-efflux pathway. Inhibition of this regulatory mechanism attenuates alkaline shifts in pH_i during capacitation as well as the ability of sperm to produce a secretory response to zona pellucida agonists. These data suggest that one aspect of mouse sperm capacitation is the selective activation of one major pH_i regulator. © 1996 Academic Press, Inc.

INTRODUCTION

Internal pH (pH_i)[2] controls a wide range of physiological processes and provides a means of integrating diverse cellular functions with metabolic or activity states (Bock and Marsh, 1988; Busa, 1986). In sperm, alkaline shifts in pH_i are associated with three physiological processes. First, elevations of pH_i initiate and modulate flagellar motility in nonmammalian sperm and, possibly, in mammalian sperm (reviewed by Shapiro, 1987; Lindemann and Kanous, 1989; Garbers, 1989). Second, the initial development of mammalian sperm fertility occurs within the female reproductive tract through the process of capacitation and is associated with elevations of pH_i (Meizel and Deamer, 1978; Working and Meizel, 1983; Vredenburgh and Parrish, 1995). Sperm fail to capacitate when alkalinization is prevented (Parrish *et al.,* 1989). Finally, sperm of many animal species must

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[2] Abbreviations used: AR, acrosome reaction; BCECF and BCECF-AM, 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein-free acid and acetoxymethyl ester, respectively; CFDA-AM, carboxyfluorescein diacetate acetoxymethyl ester; BSA, bovine serum albumin; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; H_2-DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid; DMO^-, 5,5-dimethyloxazolidine-2,4-dione; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $NMDG^+$, *N*-methyl-D-glucamine$^+$; pH_i, internal pH; SITS, 4-acetoamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; ZP, zona pellucida.

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complete the acrosome reaction (AR), a Ca^{2+}-dependent secretory event, prior to fertilization (reviewed by Yanagimachi, 1994). In mammals, the AR is controlled by adhesive contacts between sperm and the egg's extracellular matrix, or zona pellucida (ZP). ZP3, the AR-inducing glycoprotein constituent of the mouse ZP, and related agonists in other species promote exocytosis (reviewed by Wassarman, 1988; Wassarman and Mortillo, 1991). The ZP-activated signal transduction pathway includes an elevation of pH_i that is essential for the activation of voltage-sensitive Ca^{2+} channels and for exocytosis (Endo *et al.,* 1988; Florman *et al.,* 1989, 1992; Florman, 1994).

Regulation of pH_i has been explored extensively in somatic cells and is mediated by intracellular buffering systems and by transmembrane acid/base transport pathways (Roos and Boron, 1981). Three major transport mechanisms have been described and are widely distributed, including: a Na^+–H^+ exchange pathway composed of isoforms that differ in sensitivity to amiloride analogs and that are encoded by the *NHE1-4* gene family (reviewed by Fliegel and Frohlich, 1993; Tse *et al.,* 1993); a Na^+-independent Cl^-/HCO_3^- exchange mechanism that is inhibited by stilbene disulfonates and that is encoded by *AE1-3* genes (designated "anion exchanger" and reviewed by Kopito, 1990; Alper, 1991); and a Na^+-dependent Cl^-/HCO_3^- exchange mechanism that is inhibited by stilbene disulfonates and is of unknown molecular properties (reviewed by Bock and Marsh, 1988; Thomas, 1989). These pathways are reversible, although typically the two Na^+-dependent pathways utilize the inwardly directed Na^+ gradient to produce net acid export, whereas the Na^+-independent exchanger often produces acid import. In addition, specialized pH_i regulators with more restricted distributions have been characterized (Bock and Marsh, 1988).

In contrast, pH_i regulation in mammalian sperm is not well understood. A Cl^-/HCO_3^- exchange activity is present, as indicated by ion flux experiments using HCO_3^- and SO_4^{2-} radiotracers (Okamura *et al.,* 1988; Tajima and Okamura, 1990). In addition, Northern hybridization with AE2 cDNA and immunofluorescent labeling of sperm with antisera directed against AE1 and against AE2 support the notion that a Na^+-independent anion exchange pathway is present (Parkkila *et al.,* 1993). Similarly, conventional and novel transcript sizes are detected by Northern hybridization of testis poly(A)$^+$ RNA with NHE1 and NHE2 cDNA, suggesting the presence of a Na^+–H^+ exchange pathway in sperm (Orlowski *et al.,* 1992; Yu *et al.,* 1993). It is therefore presumed that both a Na^+–H^+ exchanger and an anion exchanger are present in mammalian sperm, although these activities have not been analyzed systematically and their relative contribution to pH_i regulation has not been determined.

Here, pH_i recovery following the imposition of alkaline and of acid loads is examined in cauda epididymal mouse sperm using intracellular, pH-sensitive dyes and digital image processing-enhanced fluorescence microscopy. Mouse sperm lack identifiable transport mechanisms mediating recovery following internal alkalinization. In contrast, two acid efflux pathways are described. One mechanism is dependent upon extracellular Na^+, Cl^-, and HCO_3^-, but has an ion selectivity and inhibitor sensitivity that is different from the anticipated characteristics of the somatic cell Na^+-dependent Cl^-/HCO_3^- exchange mechanism. This pathway must be functional for sperm to complete capacitation and express the ability to initiate ZP-regulated acrosomal exocytosis. The second pathway is characterized by selective inhibition by arylaminobenzoates.

EXPERIMENTAL METHODS

Incubation media. The standard media are shown in Table 1. All media also included lactate (23.28 m*M*), pyruvate (0.33 m*M*), glucose (5.56 m*M*), penicillin G (0.05 mg/ml), and streptomycin sulfate (0.05 mg/ml) and were maintained at 37°C. Further modifications of these basic media are described in the text.

Cell preparations. ZP were obtained by density gradient centrifugation of a crude ovarian homogenate and soluble extracts of isolated ZP were prepared, as described previously (Florman *et al.,* 1984, 1992). Sperm were obtained from caudae epididymides of CD-1 mice (retired breeders, Charles River Laboratories) and capacitated as described previously (Florman and Storey, 1982). Acrosome reactions were determined by a Coomassie blue binding assay (Moller *et al.,* 1990; Miller *et al.,* 1993).

Cells were immobilized on Cell Tak-coated coverslips and placed in a microscope stage microincubator (Medical Systems Corp., Greenvale, NY) to control gas phase and temperature of the sample area. Sperm were constantly superfused (0.25 ml/min) at 37°C. When using HCO_3^-/CO_2-containing media, superfusate reservoirs and peristaltic pumps were stored in a temperature- and gas phase-regulated plexigas chamber. Media was conducted through a short length (25–30 cm) of gas-impermeable tubing to the microscope stage, reducing loss of HCO_3^- during delivery of superfusate to the stage to <10% (estimated with [^{14}C]HCO_3^-).

The pH-sensitive dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was introduced prior to immobilization by incubating sperm for 15 min in media containing 6–8 μM of the membrane-permeant, acetoxymethyl ester precursor form of this dye (BCECF-AM). Sperm were immobilized and extracellular BCECF-AM and BCECF, presumably generated by esterolytic activity released from moribund sperm, were removed by superfusion. These conditions routinely produce 100–150 μM intracellular BCECF. In some cases, parallel experiments were also carried out with intracellular 5(6)-carboxyfluorescein, generated *in situ* by incubation with the permeant ester precursor form, carboxyfluorescein diacetate acetyoxymethyl ester (CFDA-AM). Alkaline loads were imposed by prepulses with 50 m*M* DMO^- (5,5-dimethyloxazolidine-2,4-dione$^-$) or with 20 m*M* propionic acid and acid loads were imposed with 10

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TABLE 1
Solutions

Component (mEq/liter)	Solutions[a] 1 HCO_3^-	2 Hepes	3 Hepes $-O\ Na^+$	4 HCO_3^- $-O\ Na^+$	5 Hepes $-Cl^-$	6 HCO_3^- $-Cl^-$	7 Sucrose
Na^+	143.3	143.3	0	0	143.3	143.3	10
K^+	4.8	4.8	4.8	4.8	4.8	4.8	0
Mg^{2+}	1.2	1.2	1.2	1.2	1.2	1.2	0
Ca^{2+}	1.8	1.8	1.8	1.8	1.8	1.8	0
$NMDG^+$	0	0	143.3	143.3	0	0	0
Cations	151.1	151.1	151.1	151.1	151.1	151.1	10
Cl^-	128.6	126.1	126.1	128.6	0	0	0
HCO_3^-	22.5	0	0	22.5	0	22.5	0
$Hepes^-$	0	25	25	0	25	0	10
$Gluconate^-$	0	0	0	0	126.1	128.6	0
Anions	151.1	151.1	151.1	151.1	151.1	151.1	10
Sucrose	0	0	0	0	0	0	230
CO_2 (%)	0	5	0	5	0	5	0
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4

[a] *Solutions 1–7* are standard media and are subsequently modified as indicated in the text.

m*M* NH_4^+ prepulses during superfusion (Roos and Boron, 1981; Thomas, 1984).

Optical techniques. pH_i of single sperm was determined from the fluorescence emission of intracellular dye, similar to the approaches described previously for determination of internal [Ca^{2+}] (Florman *et al.*, 1989; Clark *et al.*, 1993; Florman, 1994). Excitation illumination from a 100-W Hg arc was directed through 450- and 510-nm filters (20 and 23 nm bandpass, respectively; Omega Optical). Filter selection was provided by a Lamda 10 filterwheel (Sutter Instruments) controlled by Fluor-AT (Universal Imaging Corp.). Photobleaching and phototoxicity were minimized by introducing computer-actuated shutters, thereby restricting illumination to the periods of data acquisition, and by attenuating excitation light 100- and 10,000-fold with ND2 and ND4 neutral density filters, respectively. Emitted light was selected with a 540-nm long pass dichroic mirror and a 550-nm emitter filter (540DRLPO2 and a OG550, respectively; Omega Optical) and collected with a Nikon 60X PlanApo objective (1.4 NA). Images were integrated from 4–8 frames, digitized using a GenIISys-intensified Dage 72 CCD camera, and were stored and analyzed with Fluor-AT.

A nigericin/high K^+ calibration protocol was applied to derive pH_i values from fluorescence emission data (Thomas *et al.*, 1979), obtained at pH_o 7.4. Values are considered ''calculated pH_i'' in the absence of independent verification. Initial rates of pH_i change were estimated from extrapolation of the linear initial phase (typically the first minute) of recovery following imposition of acid or base loads.

RESULTS

Spectral characteristics of intracellular dye. BCECF is distributed uniformly throughout mouse cauda epididymal sperm. A large fraction (76 ± 6%, $n = 3$) of the internal dye is released following treatment with a plasma membrane-permeabilizing agent (0.01% digitonin, w/v) in a Ca^{2+}-free medium and probably represents a cytoplasmic pool. The digitonin-resistant pool of dye is located in the anterior head and in the midpiece (15 ± 3 and 9 ± 4% of total dye, respectively; $n = 3$). Dye restricted to the anterior head is released by addition of 1 m*M* Ca^{2+} to digitonin-treated sperm under conditions known to promote acrosomal secretion in sperm of other species (Noland and Olson, 1989; Florman, 1994) and is probably located within the acrosome. Finally, the BCECF located in the midpiece likely reflects dye entry into the mitochondrion. Therefore, BCECF signals derive largely, but not exclusively, from sperm cytosol.

To determine the pH-dependent spectral alterations of intracellular BCECF, sperm were treated with 5 μM nigericin, the K^+/H^+ transporting ionophore (Reed, 1979; Thomas *et al.*, 1979), and superfused with a K^+-based medium (*Solution 1* with 90 m*M* Na^+ substituted with K^+). This protocol sets pH_i equal to pH_o, as demonstrated in larger cells where direct comparisons between pH_i values obtained from dyes and from pH-sensitive microelectrodes have been reported. Comparison of spectra obtained from intracellular dye and from dye in cell-free solutions demonstrates that intracellular spectra are red-shifted by 10–12 nm. A typical shift in the normalized excitation spectrum is shown in Fig. 1 and

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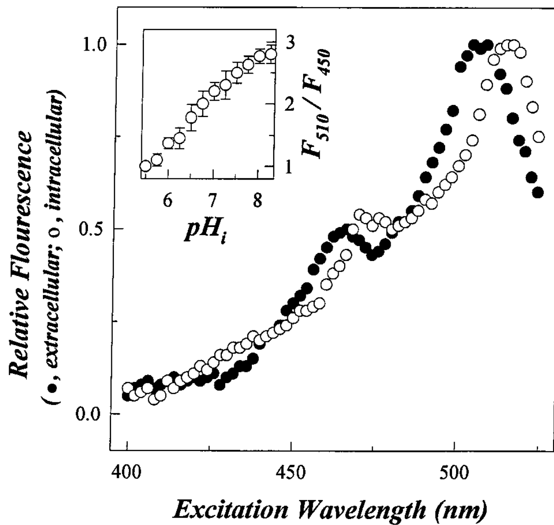

FIG. 1. Excitation spectrum of extracellular and intracellular BCECF. Extracellular spectra were obtained with the acid-free form of BCECF suspended in an "intracellular medium" (100 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 40 mM Hepes). Intracellular spectra were obtained from dye that had been passively loaded into sperm by incubation with the permeant ester precursor form and subsequently deesterified *in situ.* Spectra were obtained as described under Experimental Methods. The curves displayed were obtained at pH 7 and are representative of families of spectra obtained at pH 6–8. Note the red shift in the excitation peak of dye within sperm, relative to the free acid form of the dye in solution. (Inset) The ratio of fluorescent emission following sequential excitation at 450 and 510 nm is an indicator of mouse sperm pH_i.

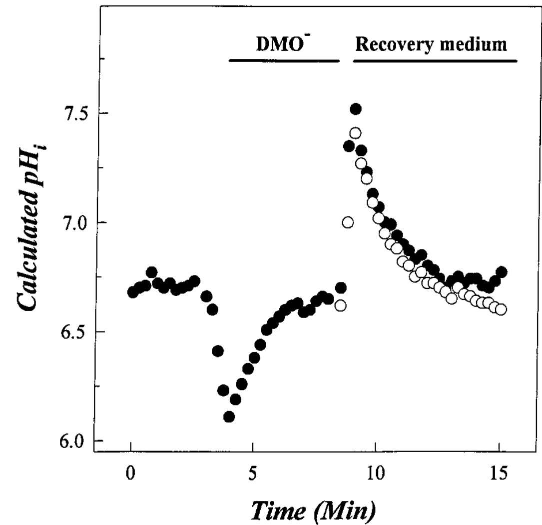

FIG. 2. Mouse sperm pH_i recovers spontaneously following imposition of alkaline loads with DMO^- prepulses. Data were obtained from single cells that were superfused with a standard HCO_3^- medium (*Solution 1*), with medium supplemented with 50 mM DMO^-, and with test media lacking DMO^-. Two representative cells demonstrate that the initial rate of pH_i recovery following alkaline loading was similar whether recovery occurred in *Solution 1* (closed symbols) or in a Hepes-buffered sucrose solution (open symbols).

the results are summarized in Table 2. Smaller red shifts have been reported in the spectrum of other fluorescein-based fluorophores within somatic cells (Chaillet and Boron, 1985) and may reflect interaction of probe with cellular proteins or membranes. The smaller cytosolic volumes in sperm (Hammerstedt *et al.,* 1978) may account for the relatively larger shifts observed here. Despite these differences, the pH_i-dependent alterations in dye fluorescent emission demonstrate that BCECF can be used as a pH_i indicator in sperm (Fig. 1, inset).

TABLE 2
Comparison of Spectral Characteristics of Intracellular and Extracellular BCECF

	Probe environment[a]	
	Extracellular	Intracellular
Peak emission	531 ± 5 (4)	543 ± 6 (6)
Peak excitation	504 ± 3 (4)	512 ± 4 (6)
Isosbestic point	441 ± 3 (4)	452 ± 3 (6)

[a] Excitation and emission spectra were acquired with an AlphaScan II fluorometer (slits, 4 nm; Photon Technology Int.). Extracellular signals were obtained from BCECF-free acid in solution and intracellular signals were obtained from dye incorporated into sperm by prior incubation with BCECF-AM, the permeant ester precursor form. Intracellular spectra were acquired from 2.5 * 10^6 sperm/ml in 1-ml volumes. Data represent the means of 4–6 separate spectra, with the number of replicates indicated parenthetically.

pH_i of cauda epididymal mouse sperm was 6.52 ± 0.05 as reported by BCECF (371 cells; all pH_i data expressed as means ± standard error), while CF reported a value of 6.61 ± 0.03 (122 cells). The different calculated pH_i values may reflect distinct dye distributions within sperm. Data presented in the remainder of this study are based on BCECF fluorescent emission and are confirmed in carboxyfluorescein experiments.

pH_i response of sperm to alkaline loads. Alkaline loads were introduced into sperm by prepulses with 50 mM DMO^-. A typical experiment is shown in Fig. 2 and the recovery rate following alkalinization was 0.58 ± 0.03 pH/min (n = 72 cells). The major pH_i regulators of somatic cells couple transport of acid equivalents to the transport of Na^+, Cl^-, or HCO_3^- (Roos and Boron, 1981; Bock and Marsh, 1988). Removal of these ions individually or in concert (*Solutions 2–6*) did not attenuate recovery rates by >10%. In fact, acid influx rates were only reduced to 0.52 ± 0.04 pH/min during recovery in a pH-buffered sucrose solution (*Solution 7*). In addition, the acid influx mechanism was unaffected by several inhibitors of acid/base transport path-

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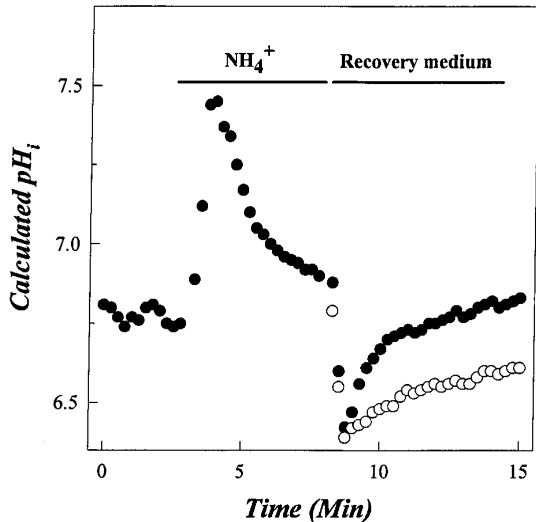

FIG. 3. Mouse sperm pH_i recovers spontaneously following imposition of acid loads with NH_4^+ prepulses. Sperm were superfused with a standard HCO_3^- medium and acid loads were produced by sequential addition and removal of 10 m*M* NH_4^+ (Roos and Boron, 1981). Two representative cells demonstrate that the initial rate of pH_i recovery in a Na^+-free medium (open symbols) is greatly attenuated, relative to recovery rates observed during incubation in a Na^+-based medium (closed symbols).

ways of somatic cells, including amiloride analogs (dimethylamiloride, ethylisopropylamiloride), arylaminobenzoate (dephenylamine-2-carboxylate, flufenamic acid), loop diuretics (bumetanide, furosemide), and stilbene disulfonates (DIDS, DNDS, SITS, H_2SITS). Thus, the mechanisms that mediate pH_i recovery following intracellular alkalinization do not require specific extracellular anions or cations and do not display a sensitivity to antagonists that is anticipated of a somatic cell acid/base transport pathway.

Effects of cations and anions on pH_i recovery of sperm from acid loads. Following intracellular acidification in response to removal of NH_4^+, cauda epididymal mouse sperm (in *Solution 1*) recover pH_i spontaneously with an initial rate of 0.31 ± 0.04 pH/min. As shown in Figs. 3 and 4A, the recovery rate was attenuated by 56 ± 8% (n = 4) when Na^+ was replaced with $NMDG^+$ or Ch^+ (representing complete and Ch^+-modified *Solution 4,* respectively). The ED_{50} for Na^+ was 30–35 m*M.*

The Na^+ specificity of this pathway was explored in two experiments with Li^+, a cation that frequently can support Na^+-dependent transport mechanisms. Li^+ did not support pH_i regulation by this transporter: ≤30 m*M* Li^+ produced no stimulation and low rates of recovery at higher $[Li^+]$ (100 m*M*) do not represent a significant stimulation (Fig. 4A). In order to explore the mechanism of Li^+ action, sperm were incubated in a modified standard HCO_3^- media (modified *Solution 1*) containing 83 m*M* $NMDG^+$ and 60 m*M* Na^+, an external $[Na^+]$ that does not saturate pH_i recovery. Acid loads were imposed by removing NH_4^+ from modified *Solution 1,* while simultaneously replacing $NMDG^+$ with Li^+. External $[Na^+]$ was held constant during this experiment. As is shown in Fig. 4A (inset), Li^+ inhibited the Na^+-dependent recovery of mouse sperm from intracellular acidification. The most reasonable explanation is that Li^+ competes with Na^+ for a binding site but is not capable of activating this acid-efflux mechanism.

Recovery from intracellular acidification also required extracellular anions. Replacement of either Cl^- or CO_2/HCO_3^- with gluconate$^-$ (Fig. 4B) or with several other anions (aspartate$^-$, SCN^-, SO_4^{2-}, Hepes$^-$; not shown) reduced recovery rates by approximately 50%. IC_{50} values of 45 ± 7 and 12 ± 4 were determined for Cl^- and for HCO_3^-, respectively. The effects of concerted depletion of external Cl^- and HCO_3^- are not additive. In three experiments, the recovery in a standard-HCO_3^- medium (*Solution 1*) was 0.33 ± 0.03 pH/min. This rate was reduced to 0.17 ± 0.04 pH/min by Cl^--depletion (*Solution 6*), to 0.14 ± 0.02 pH/min by HCO_3^--depletion (*Solution 2*), and to 0.15 ± 0.03 pH/min by the combined depletion of both anions (*Solution 5*). The major pH_i regulatory mechanism that mediates recovery

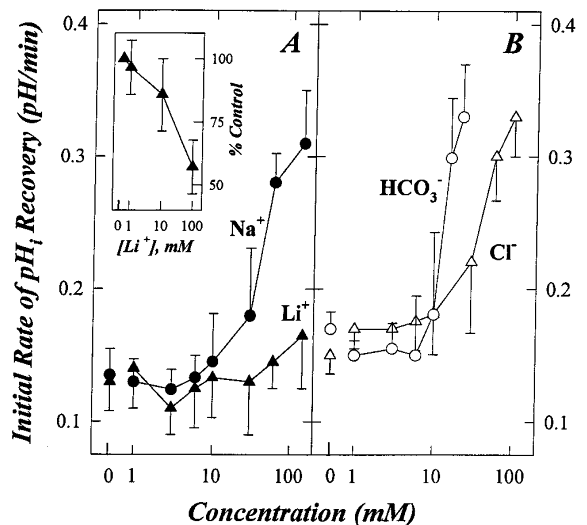

FIG. 4. pH_i recovery following the imposition of an acid load with NH_4^+ requires specific external cations and anions. Initial rates of pH_i recovery were obtained from single mouse sperm, as described under Experimental Methods and as illustrated by Fig. 3. Note the truncated scale used on the ordinate axes. (A) Recovery rates in a standard HCO_3^- medium (*Solution 1*) are attenuated by substitution of $NMDG^+$ for extracellular Na^+ (closed circles). Li^+ did not support pH_i recovery in a Na^+-free medium (*Solution 4;* closed triangles). Also shown is the inhibitory effects of Li^+ on pH_i recovery in media with a constant, 60 m*M* Na^+ (inset). (B) Recovery rates in a Na^+-containing medium are inhibited by the separate substitution of gluconate$^-$ for extracellular HCO_3^- (open circles) or for extracellular Cl^- (open triangles). Data points are the means (±SEM) of observations on 47–109 sperm.

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TABLE 3
Anion selectivity of sperm acid-export mechanism

Anion[a]	Initial pH_i recovery rate (pH/min)	
	Maximal initial rate	K_R (m*M*)[b]
Complete medium	0.37 + 0.04[c]	—
Gluconate$^-$ medium	0.16 + 0.01	—
+HCO_3^- (22.5 m*M*)	0.33 + 0.05[c]	12 ± 4
+Cl^-	0.32 + 0.02[c]	45 ± 7
+NO_3^-	0.28 + 0.02[c]	56 ± 9
+I^-	0.26 + 0.03[c]	73 ± 12
+Br^-	0.22 + 0.01[c]	88 ± 17

[a] Sperm were superfused in standard-HCO_3^- medium containing 10m*M* NH_4Cl. Acid loads were imposed by changing superfusate to media lacking NH_4Cl (complete medium, *Solution 1*) or NH_4Cl-free medium containing the indicated anion composition. Gluconate$^-$ medium is identical to standard Hepes/-Cl^- (Table 2, *Solution 5*), whereas other media are composed by substitution of 1–100 m*M* of other test anions for gluconate$^-$. Recovery rates are the means (+SEM) of 6–8 separate experiments and each experiment provided data on 8–21 separate cells.

[b] K_R is a recovery constant expressing the affinity of the Na^+-, and Cl^-- and HCO_3^--dependent pH_i regulator for anions. K_R and maximal initial rates (R_{MAX}) are obtained by normalizing recovery data for minimal (gluconate medium) and maximal rates (complete medium) and fitting normalized data to the expression $r = (R_{MAX} * [A^-])/(K_R + [A^-])$, where r is the initial recovery rate at each anion concentration ($[A^-]$). Initial rates did not attain R_{MAX} values for NO_3^-, I^-, and Br^- over the concentration range tested (1–100 m*M*).

[c] The initial rates of pH_i recovery are different than that in gluconate$^-$ medium (of the same cation composition), as determined by *t* tests of unpaired observations ($P < 0.01$).

from intracellular acidification is dually dependent on both Cl^- and HCO_3^-.

The anion selectivity of this pH_i regulator was determined by superfusing sperm with Hepes-buffered medium in which Cl^- and HCO_3^- are replaced by gluconate$^-$ (*Solution 5*). During imposition of acid loads, cells were transferred to media in which gluconate$^-$ was replaced by various test anions. Anion selectivity was determined from the initial rates of pH_i recovery in the presence of test anions. As shown in Table 3, the apparent anion selectivity of this pH_i regulatory mechanism is: $HCO_3^- > Cl^- > NO_3^- > I^- > Br^-$. The pH_i recovery from acid loads that is supported by these anions is, in all cases, greatly attenuated by replacement of external Na^+ with $NMDG^+$. Moreover, the effects of Na^+ depletion and of Cl^- depletion were not additive: initial rates were reduced by 56% following Na^+ depletion, by 49% following Cl^- depletion, and by 55% after removal of Na^+ + Cl^- (not shown). These observations are consistent with the presence of a single pH_i regulatory mechanism that accounts for about 50% of pH_i recovery following intracellular acidification and that is dependent on Na^+, on Cl^-, and on HCO_3^-.

This pathway accounts for all of the cation-dependent pH_i regulation detectable in cauda epididymal mouse sperm. In this regard, a K^+-dependent acid-efflux mechanism that may represent a voltage-sensitive proton channel (Lyall and Biber, 1994) is present in bovine sperm (Babcock *et al.,* 1983; Babcock and Pfeiffer, 1987). Yet, we observed that 60 m*M* $[K^+]_o$ had no effect on the resting pH_i or on the rate of pH_i recovery following intracellular acidification (not shown). K^+-dependent pH_i regulatory mechanisms are either absent from mouse sperm or account for only a small (<10%) component of the total acid flux.

Effects of ion transport inhibitors on the pH_i response of sperm to acid loads. Several antagonists of the acid/base transport mechanisms of somatic cells were used to define sperm pH_i regulatory pathways in greater detail. Figure 5A shows that pH_i recovery of sperm is greatly attentuated by DIDS (IC_{50} = 50 *μM,* closed circles). A second stilbene disulfonate, SITS, also inhibited pH_i recovery, although this effect exhibited a complex and poorly understood time dependence. Maximally effective concentrations of these agents inhibited pH_i recovery by about 50%. In contrast, several other stilbene disulfonate inhibitors of anion transport in somatic cells had no effect on acid efflux from mouse sperm (H_2-DIDS, DNDS). DIDS (Fig. 5A, open circles) and SITS (not shown) failed to inhibit pH_i re-

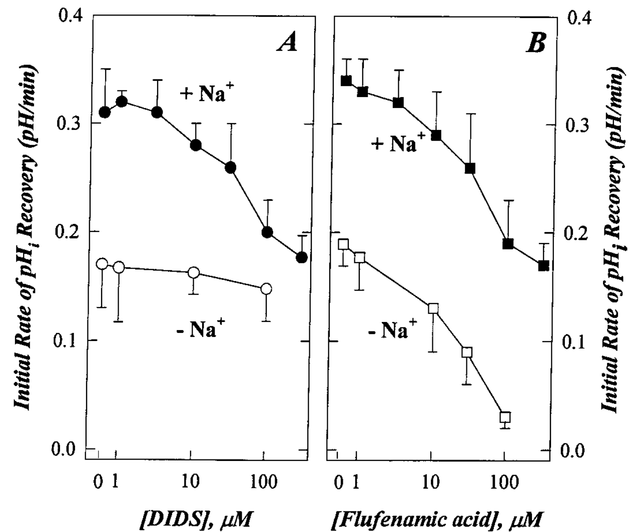

FIG. 5. Effects of DIDS and flufenamic acid on pH_i recovery following imposition of an acid load in mouse sperm. Acid loads were imposed and initial rates of recovery estimated as described under Experimental Methods and as illustrated in Fig. 3. (A) DIDS inhibits the initial rate of recovery in the presence of Na^+ (closed circles) but not in a Na^+-free media (open circles). Data represent the means (±SEM) of observations on 19–74 cells that were collected in 3–9 separate experiments. (B) Flufenamic acid inhibits the initial rate of pH_i recovery in both Na^+-based media and in Na^+-free solutions (closed and open squares, respectively). Data represent the means (±SEM) of observations on 16–59 sperm, collected in 3–7 separate experiments.

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covery in Na^+-free media, indicating that these stilbene disulfonates specifically inhibit the Na^+-, Cl^--, and HCO_3^--dependent acid-efflux mechanism of sperm.

Recovery from internal acidification was also greatly attenuated by flufenamic acid, an inhibitor of some classes of Cl^- transport mechanisms (Fig. 5B, closed squares). This arylaminobenzoate inhibited the initial rate of pH_i recovery by sperm in a standard-HCO_3^- medium (*Solution 1*) by 57 ± 6% (IC_{50} = 50–100 μM). Flufenamate also inhibits pH_i recovery in Na^+-free media (*Solution 4;* Fig. 5B, open squares), indicating that it acts specifically on that component of recovery that is not mediated by the Na^+-, Cl^--, and HCO_3^--dependent pathway. Flufenamate inhibits several Cl^- transport pathways. Yet, removal of external Na^+ also eliminates the Cl^--dependent component of sperm pH_i recovery. Thus, in these cells flufenamate selectively inhibits a Cl^--independent aspect of pH_i recovery. Diphenylamine-2-carboxylate, an arylaminobenzoate related to flufenamic acid, had similar effects although this agent was less potent. The arylaminobenzoate-sensitive pathway and the Na^+-, Cl^--, and HCO_3^--dependent regulatory mechanism together completely account for pH_i recovery from internal acidification.

Several other inhibitors of somatic cell pH_i regulatory mechanisms had no effect on recovery rates. Dimethylamiloride and ethylisoproylamiloride are 5′-substituted analogs of amiloride that are typically used as relatively selective, competitive inhibitors of Na^+–H^+ exchange (Kleyman and Cragoe, 1988). When these compounds (0.5–50 μM) were added to sperm in media of pH_o 6.5–7.5, pH_i shifted to the prevailing pH_o (not shown). A protonophore activity has been ascribed to these compounds (Davies and Solioz, 1992) and may account for this alkalinization. Inhibitors of Cl^- transport or of anion transport also failed to attenuate recovery from acid loads, including 100–500 μM concentrations of: bumetanide, furosemide, metolazone, and probenecid.

pH_i regulation during sperm capacitation. Mammalian sperm must complete the program of capacitation prior to fertilizing eggs (Austin, 1951; Chang, 1951; reviewed by Yanagimachi, 1994). Previous studies indicated that pH_i of hamster and bovine sperm increases during capacitation (Meizel and Deamer, 1978; Vredenburg and Parrish, 1995), but the transport mechanisms that produce this response were not described.

Figure 6 shows that pH_i of mouse sperm also increases during incubation under capacitating conditions. pH_i increased from 6.54 ± 0.08 (range, 6.37–6.7; n = 114 cells) to 6.73 ± 0.09 (range, 6.41–6.82; n = 91) during a 2-hr incubation. Sperm populations have completed capacitation by 1–2 hr, as indicated by the ability of sperm to initiate acrosomal secretion following addition of ZP. Populations incubated in a noncapacitating environment (BSA-free *Solution 1*) exhibited only modest increases in pH_i (6.51 ± 0.04 to 6.56 ± 0.03) and fail to undergo ZP-regulated acrosomal exocytosis (Ward and Storey, 1984).

Flufenamate (100 μM; Fig. 6) and diphenyl-2-carboxylate (not shown) had no effect on either capacitation-associated alkaline shifts or on the development of a ZP-responsive state. In contrast, DIDS (100 μM) inhibited both the pH_i alteration and the expression of ZP-dependent secretion during incubation. The related stilbene disulfonate, DNDS (100 μM), lacked inhibitory effects on either process (not shown). These observations suggest that acid efflux through the Na^+-, Cl^--, and HCO_3^--dependent pH_i regulator accounts for alterations in pH_i during mouse sperm capacitation.

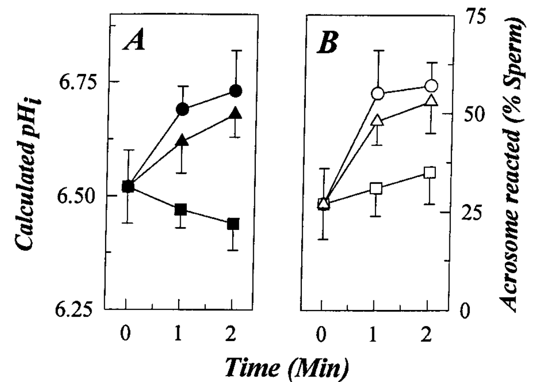

FIG. 6. The relative contribution of the Na^+-, Cl^--, and HCO_3^--dependent and the flufenamate-sensitive pH_i regulatory mechanisms in the internal alkalinization and in the expression of ZP-dependent acrosomal secretion during sperm capacitation. Sperm were incubated for 0–2 hr in a medium that supports capacitation *in vitro* (●, ○; *Solution I* supplemented with 20 mg/ml BSA) or in media containing 100 μM DIDS (■, □) or 100 μM flufenamate (▲, △). At the indicated times sperm were transferred by dilution to medium that does not support capacitation *in vitro* (2 mg/ml BSA; Ward and Storey, 1984). (A) pH_i was determined in sperm that had been preincubated for the indicated times. The pH_i increases during the first hour of incubation in capacitating media (●) or in media containing flufenamate (▲). Internal alkalinization is inhibited by DIDS (■). Data represent the means (±SEM) of observations on 34–81 sperm, collected during 3–4 separate experiments. (B) ZP-regulated acrosomal secretion is expressed during the first hour of preincubation in a capacitating medium (○) or in media containing 100 μM flufenamate (△), but is inhibited by 100 μM DIDS (□). Sperm were transferred into noncapacitating medium containing 40 μg/ml ZP glycoprotein, incubated for 30 min, and assayed for acrosomal release as described under Experimental Procedures. Data represent the means (±SD) of 3 separate experiments, with each experimental consisting of triplicate samples and with 100–200 sperm assayed/sample. Acrosomal secretion of parallel control samples (incubated with 40 μg/ml fetuin) was 21 ± 6 and 25 ± 7% after 0 and 2 hr of preincubation in capacitating media, respectively, and was not affected by either DIDS or flufenamate preincubation (not shown).

DISCUSSION

pH_i is frequently implicated in the control of such diverse functions of the mammalian sperm as the development of progressive motility, capacitation, and acrosomal exo-

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cytosis. Despite this interest, there have been no systematic examinations of the transport mechanism that control pH_i. Here, we perturbed sperm pH_i and characterized the pathways mediating recovery.

Recovery from acid loads is due to the activity of two acid efflux pathways. The first mechanism has several of the anticipated characteristics of the Na^+-dependent Cl^-–HCO_3^- exchange pathway. This conclusion is supported by a number of observations. First, the depletion of each of these three ions individually has the same effect on the initial rates of recovery as their removal in concert. In all cases the recovery rate is inhibited by approximately 50%. This is not consistent with the presence of separate anion- and Na^+-dependent mechanisms, in which case an additive effect would have been anticipated. Second, other anions support pH_i recovery in Cl^-/HCO_3^--deficient media, but only in the presence of external Na^+. This further supports the notion that Na^+, Cl^-, and HCO_3^- all act through a single transport mechanism. Finally, this Na^+-dependent mechanism is inhibited by two stilbene disulfonate inhibitors of anion transport: SITS and DIDS.

Yet, the Na^+-dependent Cl^-–HCO_3^- exchanger of somatic cells and sperm differ in several regards. Li^+ supports pH_i regulation by Na^+-dependent pathways in somatic cells and is transported by those mechanisms. In contrast, Li^+ inhibits its acid fluxes in sperm, possibly by competing with Na^+ for an extracellular binding site. Second, the anion selectivity of the sperm regulatory pathway differs from that anticipated at the somatic cell Na^+-dependent Cl^-–HCO_3^- exchange mechanism. The sperm pathway conducts Br^- at low rates and does not transport SCN^-, whereas both anions mediate robust fluxes through somatic cell exchangers. Finally, the sperm and somatic cell exchangers differ in their selective inhibition by stilbene disulfonates (Roos and Boron, 1981; Madshus, 1988; Bock and Marsh, 1988).

It therefore appears that a principal pH_i regulator which is activated by acidification of mammalian sperm has novel properties and may be a unique functional form of the Na^+-dependent Cl^-/HCO_3^- exchanger. This pathway is expected to transport Cl^- and HCO_3^- in opposite directions and during acidification should produce HCO_3^- influx coupled to Cl^- efflux. The observed inhibition of pH_i recovery in Cl^- media may reflects a rapid depletion of intracellular Cl^-. A similar effect of external Cl^- depletion has been reported in somatic cells (Boyarsky *et al.,* 1988). Direct determinations of sperm internal Cl^- during acidifications are in progress.

The Na^+-dependent Cl^-–HCO_3^- exchange mechanism, first described in the squid giant axon (Boron and De Weer, 1976) and in snail neurons (Thomas, 1977), is present in a wide range of somatic cells where it may account for a major component of the acid efflux activity (Bock and Marsh, 1988). The gene product underlying this transport pathway has not been identified. As the Na^+-dependent and -independent Cl^-–HCO_3^- exchange activities share many common features, including anion selectivity and stilbene disulfonate sensitivity, it is plausible that the former represents a functional state of an (Na^+-independent) anion exchanger (*AE*) gene product. In this regard, mammalian sperm express one or several of the *AE* genes, as demonstrated by ion flux studies, by *in situ* hybridization and Northern analysis with *AE* cDNA probes, and by immunofluorescence (Okamura *et al.,* 1988; Tajima and Okamura, 1990; Parkkila *et al.,* 1993). It is also apparent that a Na^+-independent anion exchange activity, if present, can only contribute a minor fraction (<10%) of the acid flux.

It is interesting to note that this pathway completely accounts for the Na^+-dependent pH_i regulation in mouse sperm. Alterations in external $[Na^+]$ influences several aspects of sperm function, including capacitation and spontaneous acrosomal exocytosis and it has been suggested that these effects are due to the operation of a Na^+–H^+ exchange pathway (reviewed by Fraser and Ahuja, 1988; Fraser, 1994). Yet, three types of evidence indicate that such a mechanism is either inactive or a minor pathway for acid efflux: (i) the anticipated Na^+-dependent, Cl^--independent regulatory activity is not present; (ii) Li^+ supports acid transport by the Na^+–H^+ exchanger (Seifter and Aronson, 1986; Counillon and Pouyssegur, 1993) but did not have similar effects on the sperm mechanism; and (iii) the effects of substituted amiloride analogs on sperm pH_i are complex and, despite their effects on sperm function, these compounds do not provide sufficient evidence for the participation of a Na^+–H^+ exchange pathway (Davies and Solioz, 1992).

A second acid efflux mechanism accounts for the remaining 50% of pH_i recovery following acidification and is selectively inhibited by arylaminobenzoates. These compounds were initially shown to inhibit a wide range of Cl^- transport pathways, including both stilbene disulfonate-sensitive and -insensitive systems (Greger, 1990; Cabantchik and Greger, 1992). The relationship of the arylaminobenzoate-sensitive pH_i regulator to Cl^- channels in sperm (Trezise and Buchwald, 1991; Wistrom and Meizel, 1993) and to other Cl^- transport mechanisms is a matter of great interest.

Mouse sperm pH_i also recovers spontaneously following intracellular alkalinization. In somatic cells, similar aspects of pH_i recovery are mediated by acid influx through ion-dependent transport pathways such as the Cl^-/HCO_3^- exchanger (Thomas, 1989). In contrast, the pathways that account for recovery of alkaline loads have no specific requirements for extracellular ions, for example, recovery as rapidly in a Hepes-buffered sucrose solution as in a more complex medium. Mouse sperm do not appear to utilize the types of facilitated diffusion pathways that have been well-characterized in somatic cells during recovery from alkaline loads. These cells may rely on indirect methods rather than by the types of well-characterized facilitated diffusion pathways utilized by somatic cells.

Thus, mouse sperm utilize specific membrane transport pathways to restore pH_i following internal acidification but not following alkalinization. The resting pH_i of mammalian sperm is relatively acidic compared to values routinely observed in somatic cells, as determined by indicator dyes (present study; also see Babcock *et al.,* 1983; Babcock, 1983;

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Babcock and Pfeiffer, 1987; Schoff and Lardy, 1987; Florman *et al.,* 1989; Gatti *et al.,* 1993) and by ^{31}P-NMR (Smith *et al.,* 1985), and may impose a functionally quiescent state. During storage in the cauda epididymis sperm must maintain viability and suppress spontaneous acrosomal exocytosis. An acidic pH_i contributes to the maintenance of an uncapacitated state (Parrish *et al.,* 1989) and this is associated with prolonged viability (Yanagimachi, 1994). In addition, an acidic pH_i acts as a negative regulator of sperm Ca^{2+} channels (Babcock and Pfeiffer, 1987; Florman and Babcock, 1990; Florman *et al.,* 1992), thereby minimizing unregulated Ca^{2+} entry and acrosome reactions.

This signature acidic pH_i is likely a product of the intrinsic acid load imposed continuously as a consequence of the high rate of metabolic proton generation that occurs even during epididymal storage (Cardullo and Cone, 1986) and by a decline in the ability to export monocarboxylate products of glycolysis (Garcia *et al.,* 1994). Consequently, these cells may not require specific acid-influx transport pathways to maintain acidic pH_i.

Alkaline shifts of pH_i occur during capacitation of sperm of several mammalian species (Hyne and Garbers, 1981; Working and Meizel, 1983; Vredenburgh and Parrish, 1995), including the mouse (Fig. 6). The functional consequences of capacitation, including the ability of sperm to produce ZP-regulated acrosomal secretion, are dependent upon elevation of pH_i (Fig. 6; Parrish *et al.,* 1989) and require an operational Na^+-, Cl^--, and HCO_3^--dependent pH_i regulator. Sperm may possess an anion exchanger isoform (Okamura *et al.,* 1988; Parkkila *et al.,* 1993), although this pathway is not a major regulatory mechanism in the mouse. Yet, the alternative hypothesis that this Na^+-independent mechanism is activated during capacitation and totally accounts for alkalinization is unlikely, since DNDS, the stilbene disulfonate inhibitor of the somatic anion exchanger, had no effects either on pH_i elevation or on the expression of the ZP-dependent AR. Activation of this acid efflux mechanism during capacitation is expected to produce HCO_3^- entry into sperm and may lead to the generation of pH_i-dependent or HCO_3^--dependent second messengers such as adenylyl cyclase/protein kinase A (Garbers *et al.,* 1982; Okamura *et al.,* 1991), tyrosine kinases (Visconti *et al.,* 1995, 1995), and Ca^{2+} (Garbers *et al.,* 1982; Ruknudin and Silver, 1990; Florman *et al.,* 1992; Harrison *et al.,* 1993). The mechanism by which the Na^+-, Cl^--, and HCO_3^--dependent pH_i regulator is activated is then a central issue in defining a molecular physiology of sperm capacitation.

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